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Evaluating the Effect of Chemical Treatment on DNA Recovery and Amplification from Grass Stained Cloth

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Abstract: **Grasses are the most commonly encountered botanical evidence in outdoors crime scene. Being ubiquitous in nature the grasses tend to serve as the best contact and trace evidence during a forensic investigation. Historically, these samples were identified using morphological techniques, which work well if the evidence is intact. Grasses/botanical samples are generally found in degraded, dried and contaminated forms. These types of samples lead to scarcity of vital morphological characteristics that are necessary for its correct identification. Therefore, molecular identification plays an important role in the accurate identification of the degraded and dried botanical evidence. The present study was designed as a preliminary effort in the amplification of the ITS 2 marker from the DNA extracted from chemically treated grass stains. 10 grass species were taken for the present study from six subfamilies. The stains were produced on the cotton cloth piece and were further treated with the cleaning agents (Bleach, NaOH, Soap and Gasoline). Seven out of 10 species were successfully amplified with 330-370 bp amplicons leading to 70% PCR efficiency rate. In the current study, the effect of chemicals on grass stained cotton cloth piece was studied and its impact on the recovery and amplification of ITS 2 marker.**

Index Terms: **Chemical treatment, DNA Barcoding, DNA extraction and amplification, ITS 2, Grass.**

I. INTRODUCTION

Grasses being omnipresent, and are the most commonly encountered evidence among the plant species found at the outdoor crime scene. Owing to its, morphological adaptations for seed dispersal make them the potential contact evidence that can transfer from one place to another by sticking to the clothes, foot wears or the body of victim or suspect (Ward *et al.*, 2005). Many outdoor criminal explorations involve grass as an important key to solve the crime by knowing the primary and secondary location of the corpse, route tracking of the suspect, identifying of narcotic plant etc. However, this field of forensic science has been unexplored over the years due to the lack of botanical expertise among criminal investigators. Currently, most of the botanical evidence are identified up to the genus or species level on the basis of their leaf morphology, color, size, geographic distribution and any special features such as shape and presence of trichomes or inclusion bodies etc. However, these characteristics can be used for identification only when the sample is recovered in intact and non-degraded form. Hence, the morphology-based identification methods are inconclusive in forensic cases, where samples are often compromised morphologically (CBOL, 2011). Moreover, the conventional methods are time-consuming, fallible and more subjective to human expertise (Chen *et al.*, 2010). Therefore, it is evident that DNA based approaches are superior to the identification of grass species (Grebenstein *et al.*, 1998; Ward *et al.* 2009; Saadullah *et al.*, 2016; Tahir *et al.*, 2018). Being the highly efficient, convenient and accurate technique DNA is used in a wide range of botanical evidence.

DNA barcoding is a technique in which one or shorter gene sequence is taken from a standardized portion of the genome used to identify the species (Kress & Erickson, 2008). Till now the concept of universal DNA barcode (*CO*1) can only be applied in case of animals, universal barcode in case of plants are more difficult because of the slow discriminatory rate of plant mitochondrial genome includes *CO*1.Being a candidate DNA barcode, it must have species-level genetic variability, short sequence length and conserved flanking region. Several DNA barcodes such as plastid marker *mat*K, *rbc*L, *rpo*B, *rpo*C1, nuclear marker ITS, ITS1, ITS2 and the intergenic plastidial spacers *trn*H-*psb*A, *atp*F-*atp*H and *psb*K-*psb*I have been reported (Parveen,*et al.*,2016). Depending upon its advantages

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and disadvantages, the consortium for the Barcode of Life (CBOL) has agreed upon *rbc*L and *mat*K as a core barcode region for most of the plant species. Despite this, China plant barcode of life group (CBOL, 2011) have consented on ITS2 as an alternative marker of ITS because of easy amplification and sequencing for plant species identification. Hence, in case of plants, ITS 2 marker (Nuclear genome) has been used for the identification of Fabaecae (Gao *et al.,* 2010), Poaceae (Saadullah *et al.,* 2016), medicinal plants (Gao *et al.,* 2010), Lamiceae, Dicotyledons, Monocotyledons Ferns, Mosses (Yao *et al.,* 2012) and many more. ITS 2 have short gene sequence length, easily amplified with a single pair of primers, high sequencing efficiency. ITS 2 can be used as the complimentary locus to *CO*1 for identification of plants (Chen *et al.,* 2010; Han *et al.,* 2013). ITS2 marker has conquered the limitation associated with ITS marker where PCR amplification makes multiple copies. Due to its secondary structure, it becomes a systematic tool for identification of botanical species (Han *et al.,* 2013). So, in the present study, an attempt has been carried out to amplify the extracted DNA from the chemically treated grass stains using ITS2 marker.

In order to conceal the crime, the culprit tries to wash and eliminate the botanical stains from the clothes, body of the victim even from the weapon. These will eventually deteriorate DNA both in terms of quality and quantity. Therefore, it becomes a more difficult task to analyze the evidence accurately. To carry out the study, stains on white cotton cloth from 10 grass species belonging to six subfamilies were analyzed using DNA method and the effects of chemical treatment on DNA extraction amplification was also studied.

II. Materials and Methods

A. Reagents

The phenol-chloroform extraction method (Nalini *et al.,* 2004) was used to extract the DNA from the grass stains with some modifications. The extraction buffer consists of Tris 100mM (pH 8) (Merck, Germany), EDTA 20mM (pH-8) (Merck, Germany, Urea 7M (Qualigens, Mumbai), NaCl 0.5M (Himedia, India), β-Mercaptoethanol 0.01% (10µl during the process) (BR Biochem,) and 2% SDS.

B. Sample collection

10 grass stains (10 replicas each) were produced from ten different grass species belonging to six subfamilies collected across the Punjab state in north-western India. The detailed information of selected grass species is given in Table I.

C. Species identification

The identification of grass species was done on the basis of morphological characteristics of the samples with the help of keys given by Sharma and Khosla (1989). The specific features analysed for identification were leaf (sheath, ligule and blade), inflorescence, hairs (absence or presence) and growing season (annual or perennial).

Table I: Details of grass species collected for present study

Sr.no.	Species	Sub-family
1	*Cynodon dactylon*	Chloridoideae
2	*Dactyloctenium aegyptium*	
3	*Eleusine indica*	
4	*Digiteria ciliaris*	Panicoideae
5	*Cenchrus ciliaris*	
6	*Poa annua*	Pooideae
7	*Triticum aestivum*	
8	*Aristida adscensionis*	Arundinoidae
9	*Bambusa vulgaris*	Bambusoideae
10	*Oryza sativa*	Oryzoideae

D. Stain preparation

Grass stains on white cotton cloth substrate (1cm^2) were obtained by rubbing leaf samples with moderate pressure until a visible green stain was produced.

E. Chemical Treatment

The stained cotton cloth was treated with bleach, soap, gasoline and 0.1M sodium hydroxide. Ten replicates of each species were treated with each chemical (1 cm^2 stain area in 1 ml of chemical). DNA was recovered from five replicates one hour after treatment and from the remaining five replicates after one week storage in ambient indoor conditions.

F. DNA isolation

Stained cloth piece was cut in pieces and put into the 1.5ml micro centrifuge tube. 1ml of lysis buffer and 10µl of β-mercaptoethanol were added in the tube having cloth pieces. The sample was incubated at 56°C for 7 hours. After the incubation equal amount of phenol-chloroform (25:24:1) was added and mixed well. Then, the sample was centrifuged for the separation of the aqueous phase and an organic phase. The aqueous phase was transferred into a fresh tube and chloroform: isoamyl alcohol (24:1) (BR Biochem, India) was added and mixed well. The sample was again centrifuged and the aqueous layer was transferred into a fresh tube. Sodium acetate and iso-propanol were added in the ratio 3:7. The sample tube was left undisturbed for 15 minutes at room temperature for precipitation followed by centrifugation at 4°C.The supernatant was discarded without disturbing the DNA pellet. Washing was done with 70% ethanol. DNA pellet was air-dried and preserved in TE buffer.

G. PCR primers and thermo-cycling parameters

The ITS region comprises an average 669 bp of nuclear ribosomal DNA. It includes complete sequences of ITS 1 (Internal transcribed spacer 1), 5.8S ribosomal RNA gene and ITS2. The stained derived grass DNA was checked for the PCR suitability by performing the elaborated PCR reaction using ITS 2 marker (F- 5'ATGCGATACTTGGTGTGAAT3', R-5'CCTCCGCTTATTGATATGC-3'). The total volume of PCR

mixture was 25µl which comprise 2.5µl of 10x PCR buffer, 2.5µl of $MgCl_2$ (2.5mM), 1.2µl of Taq polymerase, 2µl of dNTP's, 0.75µl of Primer (each), 13.3µl of sterilized water/molecular water, 2µl of DNA template. The optimized temperature conditions for PCR of ITS 2 are detailed in Table II. Separated amplified products were visualized under Gel-Doc EZ imager (BioRad) after the agarose gel electrophoresis in an Ethidium bromide stained 2% agarose gel.

Table II: PCR optimization conditions of DNA amplification

Steps	Temperature	Duration	Cycles	Activity
1	94°C	5 minutes	1	Initial denaturation
2	94°C	30 seconds	↑ 35	Denaturation
3	51°C	40 seconds		Annealing
4	72°C	1 minute		Elongation
5	72°C	10 minutes	↓ 1	Final elongation
6	4°C	Infinity		Storage

H. DNA Sequencing

The amplified DNA was preceded further for sequencing procedure for the accurate identification of the grass species. The sequencing of amplicons was done by Abi 3130 genetic analyzer. The DNA sequences were aligned using MEGA 6 and identified by BLASTN.

III. Results

A. Identification of Grass species

The untreated grass stains were first employed to extraction and amplification. The ITS2 marker was able to amplify in 7 out of 10 grass species (*C. dactylon, D. aegyptium, E. indica, C. ciliaris, P. annua, T. aestivum, and B. vulgaris*), giving 70% of amplification success rate. After the amplification the amplicons were subjected to sequencing for identification of respective species. It was observed that all the amplified species were able to identified accurately giving 100% success rate of sequencing. Based on the identification results the amplification was preceded on treated grass stained DNA

B. DNA extraction from chemically treated grass stains

The goal of the present study was to isolate good quality DNA from the chemically treated grass stained samples**.** In total, DNA was extracted from the cloth stained with 10 grass species of 6 subfamilies that were treated with chemicals. The extracted DNA was checked on 0.8% agarose gel (Merck, Germany) stained with ethidium bromide. The gel showed the shearing of the band in all the grass samples which indicated the presence of degraded DNA depicted in fig 1. It was also observed that the grass samples that were treated with bleach were not able to give visible DNA on agarose gel in all the species while NaOH was not able to give results in case of *Aristidia adcesnsois, Digiteria ciliaris*, and *Dactylocetium aegyptium* after one week of treatment (Fig.2). The results of extracted DNA from chemically treated grass stained cloth piece after one hour and after one week is shown in Table III. It was observed from the results that the DNA was ready for the amplification process.

C. DNA amplification from chemically treated grass stains

The DNA that was extracted from the stains treated with chemicals after one week was used for amplification of ITS2 marker. The amplicons were tested using electrophoresis on 2% agarose gel. The results showed the amplification of ITS 2marker ranging from 330-370bp depicted in fig 9-12. It was observed that the grass species such as *Eleusine indica*, *Triticum aestivumCenchrus. ciliaris*, and *Bambusa vulgaris* after treated with bleach were able to amplify ITS 2 marker. However, the species such as *Dactylocetium aegyptium* and *Cynodon dactylon* could not amplify the marker after being treated with 1M NaOH solution depicted in Table IV.

Table III: Results of extracted DNA after one hour and one week of treatment with different chemicals.

Sr. no.	Species	DNA extraction							
		One hour				One Week			
		B	N	G	S	B	N	G	S
1	*Cynodon dactylon*	-	+	+	+	-	+	+	+
2	*Dactyloctenium aegyptium*	-	+	+	+	-	-	-	-
3	*Eleusine indica*	-	+	+	+	-	+	+	+
4	*Digiteria ciliaris*	-	+	+	+	-	-	+	+
5	*Cenchrus ciliaris*	-	+	+	+	-	+	+	+
6	*Poa annua*	-	+	+	+	-	+	+	+
7	*Triticum aestivum*	-	+	+	+	-	+	+	+
8	*Aristida adscensionis*	-	-	-	-	-	-	-	-
9	*Bambusa vulgaris*	-	+	+	+	-	+	+	+
10	*Oryza sativa*	-	+	+	+	-	+	+	+

*B- Bleach, N- Sodium Hydroxide, G- Gasoline, S- Soap

Table IV: Results of PCR products using mini-barcode ITS2 from extracted DNA from selected grass species after one week of treatment.

S.N.	Species	Bleach	NaOH	Soap	Gasoline
1	*Cynodon dactylon*	-	-	+	+
2	*Dactyloctenium aegyptium*	-	-	+	+
3	*Eleusine indica*	+	+	+	+
4	*Cenchru sciliaris*	+	+	+	+
5	*Poa annua*	-	+	+	+
6	*Triticum aestivum*	+	+	+	+
7	*Bambusa vulgaris*	+	+	+	+

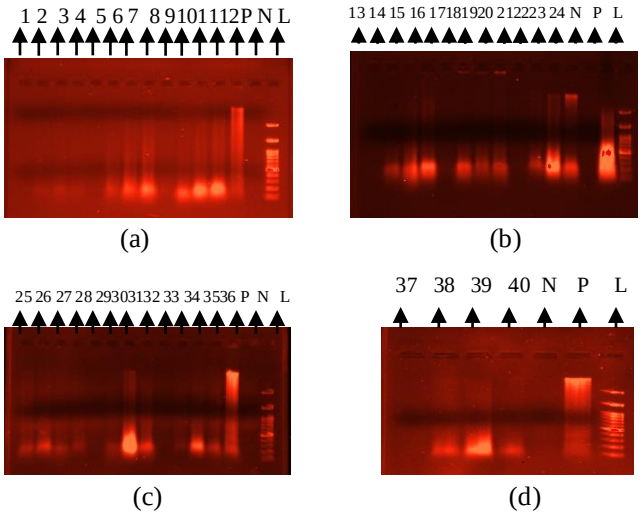

Fig 1: Results of DNA extraction after one hour of treatment with chemicals P- Positive control, N- Negative Control, L- Ladder (100bp), (a) 1- *A. adscensionis* Bleach, 2-*A. adscensionis* NaOH, 3- *A. adscensionis* Gasoline 4- *A. adscensionis* Soap, 5- *B. vulgaris* Bleach,6- *B. vulgaris* NaOH, 7- *B. vulgaris* Gasoline,8- *B. vulgaris* Soap, 9- *C. ciliaris* Bleach, 10- *C. ciliaris* NaOH,11- *C. ciliaris* Gasoline,12- *C. ciliaris* Soap. (b) 13- *T. aestivum* Bleach 14- *T. aestivum* NaOH, 15- *T. aestivum* Gasoline, 16 -*T. aestivum* Soap, 17- *O. sativa* Bleach, 18- *O. sativa* NaOH, 19- *O. sativa* Gasoline, 20- *O. sativa* Soap, 21-*E. indica* Bleach, 22- *E.indica* NaOH, 23-*E.indica* Gasoline,24-*E.indica* Soap. (c) 25- *D. aegyptium* Bleach, *26- D. aegyptium* NaOH, 27- *D. aegyptium Gasoline*, 28- *D. aegyptium* Soap, 29- *D. ciliaris* Bleach, 30- *D. ciliaris* NaOH, 31- *D. ciliaris* Gasoline, 32- *D. ciliaris* Soap, 33- *P. annua* Bleach, 34- *P. annua* NaOH, 35- *P. annua* Gasoline, 36- *P. annua* Soap. (d) 37- *C. dactylon* Bleach, 38- *C. dactylon* NaOH, 39- *C. dactylon* Gasoline, *40- C. dactylon* Soap.

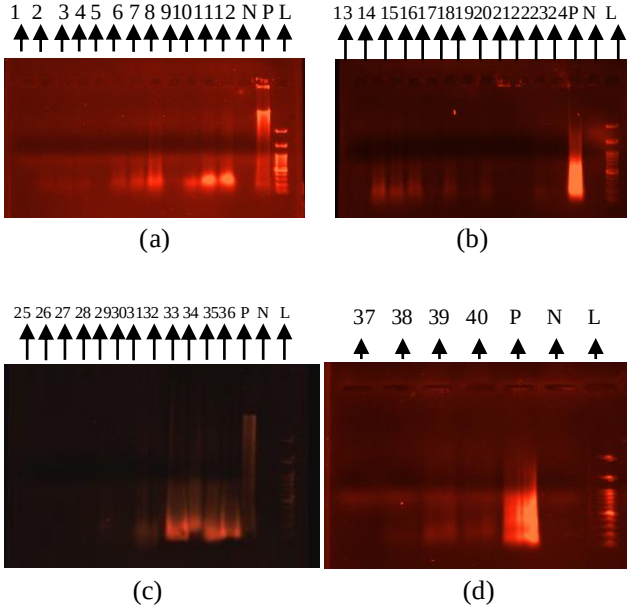

Fig 2: Results of DNA extraction after one week of treatment with chemicals P- Positive control, N- Negative Control, L- Ladder (100bp), (a) 1- *A. adscensionis* Bleach, 2-*A. adscensionis* NaOH, 3- *A. adscensionis* Gasoline 4- *A. adscensionis* Soap, 5- *B. vulgaris* Bleach,6- *B. vulgaris* NaOH, 7- *B. vulgaris* Gasoline,8- *B. vulgaris* Soap, 9- *C. ciliaris* Bleach, 10- *C. ciliaris* NaOH,11- *C. ciliaris* Gasoline, 12- *C. ciliaris* Soap. (b)13- *T. aestivum* Bleach 14- *T. aestivum* NaOH, 15- *T. aestivum* Gasoline, 16 -*T. aestivum* Soap, 17- *O. sativa* Bleach, 18- *O. sativa* NaOH, 19- *O. sativa* Gasoline, 20- *O. sativa* Soap, 21-*E. indica* Bleach, 22- *E.indica* NaOH, 23-*E.indica* Gasoline,24-*E.indica* Soap. (c) 25- *D. aegyptium* Bleach, *26- D. aegyptium* NaOH, 27- *D. aegyptium Gasoline*, 28- *D. aegyptium* Soap, 29- *D. ciliaris* Bleach, 30- *D. ciliaris* NaOH, 31- *D. ciliaris* Gasoline, 32- *D. ciliaris* Soap, 33- *P. annua* Bleach, 34- *P. annua* NaOH, 35- *P. annua* Gasoline, 36- *P. annua* Soap. (d)37- *C. dactylon* Bleach, 38- *C. dactylon* NaOH, 39- *C. dactylon* Gasoline, *40- C. dactylon* Soap

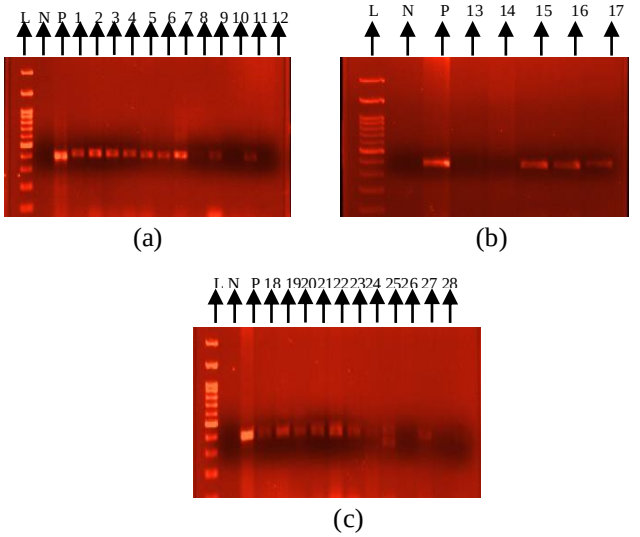

Fig 3: Results of PCR products (350bp) using mini-barcode ITS2 from extracted DNA after one week of treatment with chemicals: L- Ladder (100bp), N-Negative, P-Positive, (a) 1- *C. ciliaris* Soap , 2- *C. ciliaris*NaOH,3- *C. ciliaris* Gasoline,4- *C. ciliaris* Bleach, 5- *P. annua* Soap, 6- *P. annua* NaOH, 7- *P. annua* Gasoline, 8- *P. annua* Bleach , 9- *C.dactylon* Soap , 10- *C. dactylon* NaOH , 11*C. dactylon* Gasoline, 12*C. dactylon* Bleach.. (b) 13- *D. aegyptium* Bleach, 14-*D. aegyptium* NaOH, 15-*D. aegyptium* Gasoline, 16- *D. aegyptium* Soap, 17- *E. indica* Bleach. (c) 18- *E. indica* NaOH, 19- *E. indica* Gasoline, 20- *E. indica Soap*, 21- *T. aestivum* Soap, 22- *T. aestivum* Gasoline, 23- *T. aestivum* NaOH , 24- *T. aestivum* Bleach, 25- *B. vulgaris* Gasoline, 26-*B. vulgaris* Bleach 27- *B. vulgaris* Soap, 28-*B. vulgaris* NaOH

IV. Discussion

In recent years, the number of protocols and extraction procedures were carried out from the leaves, seeds, roots, and pollens. But, in forensic investigations, evidence are often encountered in a stained or degraded form. More or less such evidence are usually found on the cloth piece due to the friction between the plants and the substrate. However, grasses are very common corroborative evidence that can be encountered at the outdoor violence and are likely to be found on clothes, weapons and shoes of the victims or culprit during the commission of the crime. Despite all these experiments and findings very less recognition is given to the plant stained evidence. Frances *et al*, (2010) were able to extract DNA from grass stained textile in

both indoor and outdoor condition after 7, 14, and 30 days by using the standard procedure of DNA extraction. It was observed that the quantity of DNA depends on the amount of grass material transferred during the stain development procedure and also on the environmental conditions like sunlight and humidity. The similarity in the results was seen in the present study as *Aristida adscensionis* was not able to give visible DNA on agarose gel because of its thin morphological characteristic of leaves so it was impossible to produce stains on the cotton cloth. The protocol used for the present study was given by Nalini *et al.*, (2004) with few modifications during the isolation of DNA such as increase in the incubation timing from 2-3 hours to 7 hours for better lysis of cell wall. In spite of taking fresh leaf samples, chemically treated grass stained cotton cloth pieces were used for the present DNA analysis. Also, increased amount of 2-Mercaptoethanol was added just before the extraction to remove tannins and polyphenols which act as a PCR inhibitor. After all these modifications, the results showed better outcome than the standard protocol.

Another significant observation was made, that no DNA was obtained from all the samples treated with bleach. Among various chemicals used for the treatment of samples, bleach has the most adverse effect on the DNA, as it tends to degrade the DNA more rapidly as compared to the others being the strong oxidizing agent (Passi *et al.*,2012,Harris *et al.* 2006). McCord *et al.* (2011) verified the ability of HPLC-EC (High performance liquid chromatography with electrochemical detector) system to check the deleterious effect of bleach on isolated DNA from blood and buccal swab of human. The bleach chlorinates cytosine and adenine resulting in the formation of 5-Cl cytosine and 8-Cl adenine. Even in the sample treated with NaOH, having predominant O-H group, also has the ability to denature the DNA by removing the hydrogen bond between guanine and thymine by contributing protons (Wang *et al.* 2014). Apart from all the damage caused due to chlorination of DNA it does not comprise as a major factor for poor amplification of degraded samples (McCord *et al.* 2011). Same results were seen in the present study that it was possible to amplify the ITS 2 marker from the samples (one week) treated with bleach. ITS 2 marker is considered as the best marker for amplification from degraded samples. According to Han *et al* (2013), ITS2 is small in size have an ability to be a universal primer even for the degraded DNA samples as it can easily amplify that too with great success which is similar to our study most of the extracted DNA was in degraded form due to the treatment with the chemicals. It was possible to amplify the marker ranging from 330 -370bp.

The PCR amplification success rate and sequencing recoverability is very crucial factor for the barcode to be qualified as a universal barcode (Parveen *et al.*, 2016). ITS 2 marker is also considered as the universal marker for medicinal plants and its materials (Ward *et al.* 2009, Grebenstein *et al.*, 1998) Young and Colman (2004) stated that ITS 2 and its entire region is suitable, flexible and powerful for highly degraded DNA sample because it consists of conservative region 5.8s region between ITS 1 and ITS 2. Moreover, the current work also complements the results of Birch *et al.* (2017) where ITS 2 marker is considered as the informative marker for DNA barcoding in grasses. The results of our research work are somewhat similar to the work of Tahir *et al.* (2018) and Al-juhnani (2019) where the universality of ITS 2, *Rbc*l and *Mat*K marker on fresh and dry samples of medicinal species of Fabaceae and Poaceaewere observed. In both the cases where ITS 2 marker gave 360 bp (Fabaceae), 365bp (Poaceae) and 201-304 bp (dryplant) of amplicons another work done by Moorhouse-Gunn *et al.* (2018) shows the results of 291 bp (Table V).

Table V. PCR amplicon size in different studies

Sr. No.	Botanical samples	Amplicon size (bp)	References
1	Old and medicinal plants	233	Han *et al.* (2013)
2	Fabaceae	205-249	(Gao *et al.*, 2010),
3	*Avena sativa*	211	Grebenstein *et al.*, (1998)
4	*Helictotrichonconvolutum*	220	
5	Dryland plants (including Poaceae)	201-348	Al- juhnani (2019)
6	Poaceae	291	Moorhouse-Gunn *et al.* (2018)
7	Fabaceae	360	Tahir *et al.* (2018)
8	Poaceae	365	
9	Ginseng	420	Moorhouse-Gunn *et al.* (2018)
10	Dicotyledons	221	Yao *et al.*, (2012)
11	Monocotyledons	236	
12	Gymnosperms	240	
13	Ferns	224	
14	Mosses	260	
15	Poaceae	330-370	Present Study

CONCLUSIONS

The effect of chemicals on the extraction and amplification of grass stained cotton cloth piece was observed. In the present study, it was concluded that ITS 2 marker can be used for the amplification even from highly degraded samples. As, it was possible to amplify the samples treated with bleach and NaOH, in spite of their deleterious effect on DNA. The present study also supports the fact that completion of DNA analysis process depends primarily on the amount of plant material transferred to the substrate and the condition during the exchange of the material. Therefore, this work will play an important role in establishing its utility in forensic investigations.

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